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A THESIS FOR THE DEGREE OF
MASTER OF SCIENCE IN FOOD AND NUTRITION

Effects of *Doenjang*
on oxidative stress and inflammation
in adipose tissue of mice fed a high fat diet

고지방식이를 섭취한 마우스 지방조직에서
된장이 산화스트레스 및 염증에 미치는 영향

February, 2015

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Abstract

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Obesity, now regarded as chronic, low-grade inflammation, is associated with metabolic syndrome. Especially, adipose tissue is considered as a main organ leading to systemic inflammation in obesity *via* secretion of pro-inflammatory adipokines. *Doenjang*, a traditional Korean fermented soybean paste, has been reported to have the anti-obesity and anti-inflammatory effects in serum and liver. In spite of its importance in obesity-related inflammation, there is no study to investigate the anti-obesity and anti-inflammatory effects of *Doenjang* in adipose tissue. Therefore, the present study investigated the protective effect of *Doenjang* on the inflammation and oxidative stress in adipose tissue of diet-induced obesity mouse. At 6 weeks of age, male C57BL/6J mice were fed a low fat diet (LF),

a high fat diet (HF: 45% fat and 1% cholesterol), an HF containing *Doenjang* diet (HFDJ: 14.4% freeze-dried *Doenjang*) or an HF containing steamed soybean diet (HFSS: 11.7% freeze-dried steamed soybean) for 11 weeks. At the end of experiments, body weight and adipose tissue weight of mice fed an HFDJ diet exhibited significantly 16% and 19% lower than the HF group, respectively. Although there were no significant differences in adipocyte size and number among the HF diet-fed groups, consumption of *Doenjang* alleviated the incidence of crown-like structures in adipose tissue. Consistently, we observed that mice fed an LF diet and an HFDJ diet significantly lower mRNA levels of oxidative stress markers (heme oxygenase-1 and p40^{phox}), pro-inflammatory adipokines (tumor necrosis factor alpha and macrophage chemoattractant protein-1), macrophage markers (CD68 and CD11c), and a fibrosis marker (transforming growth factor beta 1) in adipose tissue than mice fed an HF diet. Gene expression of anti-inflammatory adipokine, adiponectin was significantly higher in the HFDJ and HFSS groups than the HF group. These results demonstrate that *Doenjang* may ameliorate systemic inflammation and oxidative stress in obesity *via* inhibiting inflammatory signals in adipose tissue. The anti-oxidative stress and anti-inflammatory effects observed in adipose tissue with an HFSS diet were not as significant as those with an HFDJ diet. It suggests that the bioactive compounds produced during fermentation and aging process may be involved in the observed health-beneficial effects of *Doenjang*.

Key words: adipose tissue, *Doenjang*, inflammation, obese mice, oxidative stress

Student Number: 2013-21501

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List of abbreviations

CLS: crown-like structure

COLA1: collagen, type I, alpha I

ECM: extracellular matrix

H&E: hematoxylin and eosin

HF: high fat diet

HFDJ: high fat containing *Doenjang* diet

HFSS: high fat containing steamed soybean diet

HO-1: heme oxygenase 1

HSC70: heat shock cognate protein 70

JNK: c-Jun N-terminal kinases

LF: low fat diet

MCP-1: monocyte chemotactic protein 1

MMP: matrix metalloproteinase

NOX: NADPH oxidase

ROS: reactive oxygen species

RPL19: ribosomal protein L19

TAE: tannic acid equivalent

TGF- β 1: transforming growth factor beta 1

TNF- α : tumor necrosis factor alpha

WAT: white adipose tissue

Introduction

Obesity and metabolic syndrome

The prevalence of obesity has considerably increased and has become a serious global health problem in recent decades. Obesity, particularly excess visceral adiposity, is accompanied by a chronic, low-grade inflammation, which contributes to the development of metabolic dysfunction, such as dyslipidemia, insulin resistance, non-alcoholic steatohepatitis and cardiovascular diseases (1). Therefore, it is necessary to reveal the physiopathological mechanism involved in adipose tissue enlargement and related metabolic disorders. White adipose tissue (WAT) has been traditionally considered as the main energy repository in the body; however, it is now established as an endocrine organ communicating with wide organs *via* complex networks of endocrine signals (2). The biology of WAT is substantially changed in obesity. Factors including cellular stress such as inflammation and oxidative stress are part of the biological alterations attracting and maintaining for immune cells within the WAT, influencing the remodeling of the WAT such as fibrosis and promoting inflammatory status. These biological alterations of WAT affect metabolic dysfunction of other organs by endocrine signals during obesity.

Progress of adipose dysfunction in obesity

Inflammation in adipose tissue

In the development of obesity, excessive lipids are accumulated in enlarged WAT, and then hypertrophic adipocytes trigger cellular stress including inflammation, oxidative stress and fibrosis within WAT (3). Especially, the link between obesity and its pathophysiological effects is associated with macrophage accumulation in adipose tissue (3), (4). Monocyte chemoattractant protein 1 (MCP-1) is activated to recruit macrophage into adipose tissue. Necrotic dead adipocytes are surrounded by an aggregation of single or fused macrophages, forming a characteristic histological feature “crown-like structure (CLS)”. In addition, M1 phenotype (classically activated macrophage) of infiltrated macrophages highly secretes pro-inflammatory adipokines, including tumor necrosis factor alpha (TNF- α), interleukin (IL)-6 and IL-1 β . TNF- α activates nuclear factor kappa B signaling and c-Jun N-terminal kinases (JNK) signaling, which stimulate the release of pro-inflammatory adipokines (5). In addition, TNF- α negatively regulates the expression of adiponectin, an anti-inflammatory and insulin sensitizing adipokine (6). Tissue inflammation has been shown to be tightly associated with the accumulation of extracellular matrix (ECM) components, which leads to the dysfunction of various organs, such as the liver, heart and kidney (7). Especially, CLS in the adipose tissue develops into sites of fibrosis (8). Therefore, it is becoming increasingly evident that a localized inflammation within adipose

tissue can lead to an overall systemic inflammation, which contributes to the development of obesity-linked complications (3).

Previous study showed that TNF- α expression was increased in adipose tissue of obese mice, whereas that in other tissues including liver and skeletal muscle, was undetectable in both lean and obese mice (9). In addition, deletion of macrophage in adipose tissue of diet-induced obesity mice leads to improve insulin sensitivity and to ameliorate hepatic steatosis (10). Considering these observations, it seems reasonable that initiation of obesity associated with a low grade-inflammation is originated in adipose tissue (11).

Oxidative stress in adipose tissue

An increase of oxidative stress in WAT is an early agitator of metabolic syndrome. Increase in reactive oxygen species (ROS) causes an irreversible damage to the macromolecules in cells (12). ROS can be generated by various intracellular enzymes. NADPH oxidase (NOX) is a family of enzymes that transfer electrons from NADPH to oxygen, producing superoxide (O_2^-) and H_2O_2 . NOX2 requires the combination of at least five additional components for its activation. Other NOX isoforms vary in their requirements of these components for their activation. The additional proteins involved in NOX activation include the membrane-bound $p22^{phox}$, the cytosolic proteins including $p47^{phox}$, $p67^{phox}$, the small GTPase Rac, and $p40^{phox}$, which together lead to the activation of the NOX enzyme.(13), (14). According to previous studies, NOX is a major source of ROS in pre-

adipocytes, and the elevated expression of NOX subunits was observed in obese adipose tissue of rodent and human with insulin resistance (15), (16). Consequently, the elevated ROS induces chronic inflammation by inflammatory adipokines such as TNF- α , IL-6 (17) and MCP-1 (13). In addition, infiltrated macrophages contribute to increased NOX activation and raised ROS production in obese adipose tissue (13).

Oxidative stress is associated with decreased antioxidant agents as well as increased ROS production. The decreased activity of antioxidant enzymes such as catalase, superoxide dismutase and glutathione peroxidase results in aggravation of oxidative stress. (18), (19). On the other hand, several studies showed a contrary result (20), (21). The studies argued that the antioxidant enzyme activity is increased under oxidative stress, in order to scavenge the increased ROS level.

Fibrosis in adipose tissue

Fibrosis is a common pathological consequence of many inflammatory diseases such as idiopathic pulmonary fibrosis, liver cirrhosis, systemic sclerosis, and progressive kidney disease (22), (23). Fibrosis is induced by the excessive accumulation of ECM components, including collagen I, III and VI. A high level of ECM flexibility facilitates adipose tissue expansion in healthy manner. Over the progress of the obesity, increased interstitial fibrosis in WAT may reduce ECM flexibility (24).

Matrix metalloproteinase (MMP), a family of extracellular endopeptidase, has long been considered to be mainly responsible for degradation of ECM components. However, it is now recognized that MMP has both inhibitory function including MMP1 and MMP2, and stimulatory function including MMP3, MMP9 and MMP12 in fibrosis (25).

An abnormal collagen deposition is closely related to tissue inflammation characterized by infiltration of macrophages (26). Transforming growth factor beta 1 (TGF- β 1) is an important regulator of fibrosis. MMP 9, secreted by macrophage, is crucial to activate TGF- β 1 by cleaving precursor TGF- β 1 (27). In obese subjects, over expression of MMP 9 has been observed in WAT (28), (29).

Collectively, we summarized that potential mechanisms leading to adipose tissue inflammation, oxidative stress and fibrosis in obesity based on previous studies (**Fig. 1**).

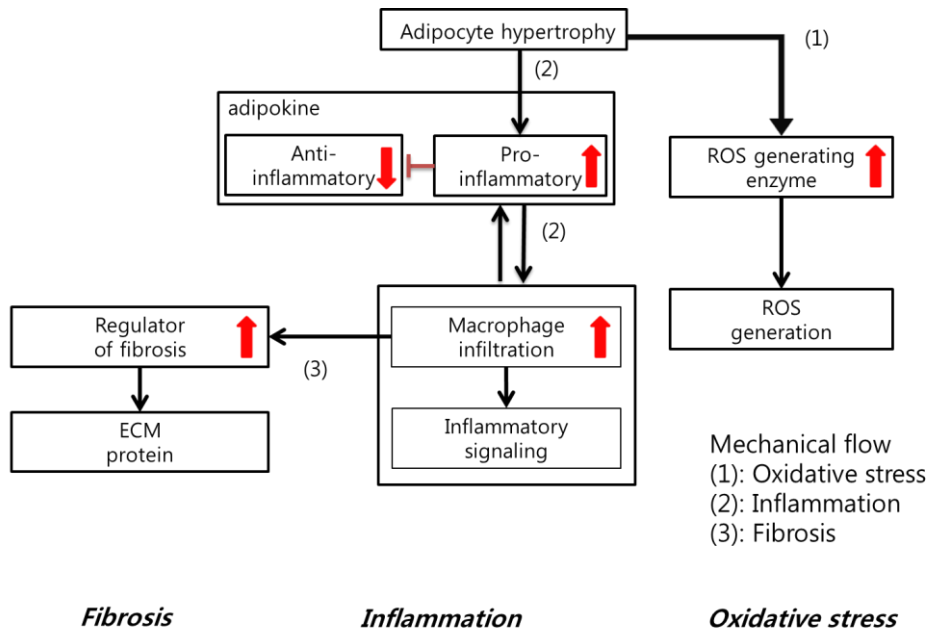


Figure 1. Potential mechanisms leading to the inflammation, oxidative stress and fibrosis in adipose tissue of obesity.

Lipid accumulation leads to adipocyte hypertrophy. Hypertrophic adipocytes activate mechanical stress such as (1) oxidative stress by activating ROS generating enzyme and (2) secrete pro-inflammatory cytokines. Pro-inflammatory adipokines stimulate infiltration of M1 type macrophages, which activate inflammatory signaling activating secretion of pro-inflammatory adipokines, and suppress anti-inflammatory adipokines. (3) In addition, infiltrated macrophages stimulate fibrosis *via* activating fibrosis regulator and accumulating excessive ECM protein in adipose tissue. Eventually, local inflammation worsens and propagates systemically *via* adipokines.

Health-beneficial effects of *Doenjang*

Anti-obesity effect of Doenjang

Doenjang is one of the traditionally used basic seasonings in Korea. It is made by three steps. Firstly, dried soybean block (*Meju*) from cooked soybean has been fermented for 1 or 2 months. Secondly, *Meju* is ripened for 1 or 2 months in jars by adding a salt solution for liquid fermentation, and then the supernatant liquid (*Kanjang*) is decanted and the remaining soy paste (*Doenjang*) finally is aged for various periods (30).

Previous studies have shown that fermented soybean foods contain higher bioactive compounds (31), (32), and exhibited stronger anti-oxidative (33) and anti-cancer activities (34), (35) than unfermented ones. Most isoflavone in cooked and in unfermented soybean exist in the glucoside form. During the process of fermentation, the cleavage of β -glycosyl bond of isoflavone glucoside by the rapid growth of microbes results in higher contents of isoflavone aglycones, including genistein and daidzein. Recently, the functionality of various fermented soybean foods has been extensively studied in both rodents and humans. Dietary feeding of *Doenjang* markedly suppressed the body weight gain, and serum oxidative stress and adipokine levels in high fat-fed mice (33), (36), (37). Taking an average daily 9.9 g freeze-dried *Doenjang* as pills for 12 weeks results in reduction in body weight and body fat mass of overweight adults (38).

Bioactive compounds of Doenjang

Recently, various health food products are supplemented with soy protein, and many countries allow health claims for foods rich in soy protein. Previous study showed that rats fed a soy protein has an effect on reducing the serum level of cholesterol, leptin and FFA level, comparing with those fed a casein protein. In addition, soy protein diet makes the size of adipocyte smaller and lessens the increase of weight (39), (40). Other study showed that feeding soy protein increases the adiponectin expression and decreases the plasminogen activator inhibitor-1 expression without change of body weight in rats (41). These beneficial properties of soy protein may due to its amino acids, soy peptides and phenolic compounds, which are isolated from soy protein from processed products or fermentation.

Soybean is enriched with amino acids such as leucine (23.6%), valine (12.22%), lysine (10.01%), isoleucine (8.8%), and phenylalanine (8.75%). Especially, soybean peptides, containing aspartate (4.74%) and glutamate (5.55%), support in performing exercise (42) and delaying fatigue (43).

Soybean-derived bioactive peptides have various health-beneficial effects, including hypocholesterolemic effect (Ile-Ala-Glu-Lys) (44), (45), (46), anti-inflammatory activity (lunasin) (47), (48), anti-oxidant activity (Leu-Leu-Pro-His-His) (49), (50), (51) and anti-obesity effect (41).

Naturally, such soy bioactive peptides are inactive within the

sequences of compact proteins. However, limited hydrolysis such as fermentation process may dissociate the compact protein structures and thereby expose the amino acid residues (52).

Besides soy peptide, content of phenolic compounds including bioflavonoids (anthocyanins, flavanols, flavonols, flavones, flavanones, isoflavones and proanthocyanins), coumestanes, lignans and stilbenoids is approximately 4~5 mg tannic acid equivalent (TAE)/g in soybean (53), (54). These phenolic compounds have been shown to be effective in cancer (55), (56) and oxidative stress (57), (58), (59). Phenolic compounds present in plants can be sorted into free or bound form: free phenolic compounds found in the vacuoles of plant cells, and bound phenolic compounds linked to cell wall structure components through several covalent bounds. The change in the profile of phenolic compounds during fermentation is due to the action of cellulolytic, ligninolytic and pectinolytic enzymes, mainly produced by the microorganism. The enzymes produced by the microorganism release phenolic compounds by breaking down the cell wall matrix. Thus, the free phenolic compounds as well as bound forms are released more efficiently from the plant matrix. Consequently, fermentation has been considered as the optimum process to obtain many phenolic compounds extracts with a high activity (60).

Doenjang contains various isoflavones as major phenolic compounds, which mainly existed in aglycone form such as genistein, daidzein and glycerin. In general, total isoflavones in soybeans are 37%

daidzein, 57% genistein and 6% glycitein, according to USDA data. Genistein, a phytoestrogen found in fermented soybeans, has variety of pharmacological features including anti-obesity, anti-inflammation, anti-oxidative stress, anti-fibrosis, anti-cancer and neuroprotection. Genistein has been shown to be effective in preventing production of NF- κ B dependent cytokines such as IL-6, IL-8 and TNF- α (61). Genistein also shows anti-fibrotic effects by decreasing collagen synthesis induced by TGF- β 1 *in vitro* (62). Besides anti-inflammatory and anti-fibrotic properties, other reported anti-obesity effects of genistein, such as impact in decreasing body and adipose tissue weight (63), (64), (65). Reportedly, genistein has antioxidant activity in Caco-2 cell via induction of phase II enzymes, heme oxygenase (HO-1) and γ -glutamylcysteine ligase catalytic (66) and other reported that feeding low dose genistein (1 mg/kg in diet) accomplished neuroprotection in global cerebral ischemia *via* elevating of Nrf2 activity and HO-1 expression (67). Daidzein as well as genistein is a major isoflavone in soybean. According to previous study, body weight of mice fed a high fat diet with daidzein supplementation was significantly decreased compared to that of mice fed a high fat diet (68). However, its anti-oxidant activity is still contradictory. After treatment of cells with daidzein, oxidative stress in chicken ovarian germ-somatic cell was attenuated (69), whereas mild oxidative stress in H4IIE Cells1 was exerted (70).

Aim of this study

The objective of this study was to determine whether *Doenjang* would alleviate inflammation, oxidative stress and fibrosis and to understand the mechanism underlying anti-inflammatory, anti-oxidative and anti-fibrotic actions of *Doenjang* in adipose tissue of mice fed a high fat diet.

Materials and methods

Animals and diets

Male C57BL/6J mice at 4 weeks of age were purchased from Nara Biotech Co. (Korea). After about 9 days of acclimation period, mice were randomly divided into 4 groups and each group was fed the respective diets (Unifaith Inc., Korea) for 11 weeks; a low fat diet (LF, n = 12), a high fat diet (HF: 45% fat and 1% cholesterol, n = 12), a high fat containing 14.4% freeze-dried *Doenjang* diet (HFDJ, n = 11) and a high fat containing 11.7% freeze-dried steamed soybean diet (HFSS, n = 12). The dose of *Doenjang* is based on a previous study, which showed that feeding 20% *Doenjang* for 8 weeks is effective in anti-obesity (37). In an HFSS diet, 11.7% of steamed soybean was added to adjust the soy protein intake to the level of a HFDJ diet. Macronutrient contents in an HFDJ and an HFSS diets were adjusted to those in an HF diet by addition of casein, soybean oil, corn starch and fiber. Traditionally prepared *Doenjang* (aged for 6 months) and steamed soybean were obtained from Institute of Sunchang Fermented Soybean Products (Korea), and were freeze-dried, powdered and stored at -20°C. Freeze-dried *Doenjang* and steamed soybean were analyzed for nutritional composition and isoflavone contents by Korea Food Research Institute and Research Institute for Food Safety at Optipham Co. (Korea). The composition of diets and the contents of bioactive compounds of supplementations

are given in **Table 1** and **Table 2**, respectively. Animals were maintained in a temperature ($21 \pm 2^{\circ}\text{C}$) and humidity ($50 \pm 20\%$)-controlled environment with a 12 h dark-light cycle, and had ad libitum access to their respective food and water throughout the study. The experimental protocol was approved by the Chungbuk National University Institutional Animal Care and Use Committee (CBNUA-636-13-01). After overnight fasting, mice were sacrificed by CO_2 asphyxiation. Blood was collected by cardiac puncture and tissues were removed, quickly frozen in liquid nitrogen and stored at -80°C until analysis.

Table 1. Composition of experimental diets

Component (g)	LF	HF	HFDJ	HFSS
Casein	200.0	200.0	152.9	152.9
Corn starch	376.7	151.0	131.95	132.71
Maltodextrin	100.0	100.0	100.0	100.0
Sucrose	172.8	200.0	200.0	200.0
Lard	–	188.5	188.5	188.5
Soybean oil	50.0	50.0	22.7	26.75
Cholesterol	–	10.0	10.0	10.0
Cellulose	50.0	50.0	36.05	27.46
AIN 93 mineral mix	35.0	35.0	35.0	35.0
AIN 93 vitamin mix	10.0	10.0	10.0	10.0
Choline bitartrate	2.5	2.5	2.5	2.5
L-cystine	3.0	3.0	3.0	3.0
<i>Doenjang</i> , freeze-dried ¹⁾	–	–	150	–
Steamed soybean, freeze-dried ²⁾	–	–	–	118
Total	1000	1000	1042	1006
Energy (kcal/g)	3.85	4.75	4.56	4.72

LF: low fat, HF: high fat, HFDJ: HF containing *Doenjang*, HFSS: HF containing steamed soybean.

1) contains carbohydrate 12.7g, protein 31.4g, lipid 18.2 g and fiber 9.3 g/100 g

2) contains carbohydrate 15.5g, protein 39.9g, lipid 19.7g and fiber 19.1 g/100 g

Table 2. Isoflavone content of freeze-dried *Doenjang* and steamed soybean

Component (mg/g)			<i>Doenjang</i>	Steamed soybean
Total polyphenol ¹⁾			15.59	3.07
Total isoflavone			1.06	3.01
Glucoside	Daidzin	Free	–	0.42
		Malonyl–	–	0.53
		Acetyl–	0.04	0.06
		Total	0.04	1.01
	Glycitin	Free	–	0.20
		Malonyl–	–	0.17
		Acetyl–	–	0.01
		Total	–	0.38
	Genistin	Free	–	0.52
		Malonyl–	–	0.90
		Acetyl–	–	0.07
		Total	–	1.49
	Total		0.04	2.88
	Aglycone	Daidzein	0.39	0.05
		Glycitein	0.16	0.02
		Genistein	0.51	0.06
		Total	1.06	0.13

1) Tannic acid equivalent (TAE)

Histological analysis

Formalin-fixed adipose tissue was processed into 4- μ m-thick paraffin sections and stained with hematoxylin and eosin (H&E) for histological evaluation. The morphology was observed under an Olympus BX50 microscope with DP-72 digital camera (Olympus, Japan) and the image was captured using Image-Pro Plus ver. 4.5 program (Media Cybernetics Inc., USA). The size and numbers of adipocytes per each field of view were measured at 200 \times magnification.

Total RNA extraction and quantitative real-time PCR analysis

The total RNA of mouse epididymal adipose tissue was isolated using the RNAiso Plus (Takara Bio Inc., Japan) and cDNA was synthesized with Superscript[®] II Reverse Transcriptase (Invitrogen, USA). mRNA levels were analyzed by quantitative real-time PCR (qPCR) using a StepOne[™] Real Time PCR System (Applied Biosystems, USA) using the SYBR[®] Green PCR Master Mix (Applied Biosystems) according to the supplier's protocol. Mouse ribosomal protein L19 (RPL19) was used as a reference gene and relative gene expression levels were analyzed using the $2^{-\Delta\Delta C_t}$ method. **Table 3** depicts the primer sequences.

Table 3. Primer sequences for qPCR

Primer	Forward (5'-3')	Reverse (5'-3')
TNF- α	GGCTACAGGCTTGRCACCTCGA	CACGCTCTTCTGTCTACTGAA
MCP-1	CCAGCACCAGCACCAGCCAA	TGGGGCGRRAACTGCATCRGGC
CD68	GCACAGCCAGCCCTACGA	GAGCTGGRGTGAACTGTGACATTT
CD11c	CTGGATAGCCTTTCTTCTGCTG	GCACACTGTGTCCGAACTC
TGF- β 1	CACCGGAGAGCCCTGGATA	TGTACAGCTGCCGCACACA
COLA1	GCTCCTCTTAGGGGCCACT	CCACGTCTCACCATTGGGG
HO-1	CCTCACTGGCAGGAAATCATC	CCTCGRGGAGACGCRRRACATA
Adiponectin	CCGTTCTCTTCACCTACGAC	TCCCCATCCCCATACAC
RPL19	TCAGGCTACAGAAGAGGCTTGC	ATCAGCCCATCCTTGATCAGC

Total protein extraction and immunoblotting

The epididymal tissues (~100 mg) were homogenized in 300 μ L of ice-cold protein lysis buffer [50 mM Hepes-KOH (pH 7.5), 150 mM NaCl, 1 mM EDTA (pH 8.0), 2.5 mM EGTA (pH 8.0), 1 mM NaF, 10 mM β -glycerophosphate, 0.1 mM Na₃VO₄, 1 mM DTT, 1% NP-40, 10% glycerol, 0.2 mM PMSF and Protease inhibitor cocktail (Sigma, USA)] using the Tissue Lyser system (Qiagen, USA) with 5 mm sterile stainless steel beads. After centrifugation for 30 min at 10,000 \times g at 4°C, the protein content of the supernatant was measured using protein assay kit (Bio-Rad, USA). Equal amounts of protein were loaded into the lanes of an SDS-PAGE gel, separated and then transferred to a PVDF membrane using a semi-dry electrotransferring unit (Bio-Rad). After blocking with 5% nonfat milk or bovine serum albumin in TTBS, membranes were probed with specific antibodies diluted in TTBS with 5% nonfat milk or bovine serum albumin as follows: anti-catalase (ab-16731, Abcam, USA), anti-HO-1 (sc-10789, Santa Cruz Biotechnology, USA), anti-p-JNK (s-9251, Cell signaling, USA), anti-70-kDa heat shock cognate protein (HSC70; Santa Cruz Biotechnology) or anti-beta-actin (A5441, Sigma, USA). The membranes were then incubated with an IgG-peroxidase-conjugated secondary antibody for chemiluminescent detection. The band intensities were quantified using Quantity One software (Bio-Rad).

Statistical analysis

Data were analyzed using SPSS software (ver. 21.0, SPSS Inc., USA). For all experiments, one-way ANOVA followed by Duncan multiple range test, was employed to assess the statistical significance. Data were expressed as the mean \pm SEM and differences were considered statistically significant at $P < 0.05$. Correlations between two variables were determined by Pearson's correlation coefficient.

Result

Effects of Doenjang on body and adipose tissue weights in mice fed a high fat diet

There were no statistical differences in the initial body weights of all groups (**Table 4**). At the end of the experiment, mice fed an LF and an HF containing *Doenjang* diet gained body weight 21% and 16% less respectively than those fed an HF diet. The body weight of mice was significantly lower in the LF and the HFDJ group than in the HF group from week 1 (**Fig. 2**). Intake of food and calorie was significantly increased in the LF group compared to mice fed HF diet-fed groups. Consistently, consumption of *Doenjang* significantly reduced epididymal, retroperitoneal and perirenal adipose tissue weights in mice fed an HF diet. Consumption of steamed soybean did not significantly change both body and adipose tissue weights.

Table 4. Body weight and adipose tissue weight of mice fed a high fat diet (HF) and HF containing *Doenjang* (HFDJ) or steamed soybean (HFSS).

	LF	HF	HFDJ	HFSS
Body weight (g)				
Initial	18.7±0.2 ^a	18.7±0.3 ^a	18.7±0.2 ^a	18.7±0.2 ^a
Final	29.3±0.7 ^a	38.0±1.0 ^b	31.8±0.8 ^a	38.8±0.7 ^b
Adipose tissue weight (g)				
Epididymal	0.87±0.08 ^a	2.04±0.07 ^c	1.65±0.12 ^b	2.14±0.08 ^c
Retroperitoneal	0.21±0.03 ^a	0.56±0.04 ^c	0.43±0.04 ^b	0.55±0.03 ^c
Perirenal	0.09±0.01 ^a	0.20±0.01 ^c	0.14±0.01 ^b	0.19±0.01 ^c
Food intake (g/day)	3.93±0.10 ^b	2.60±0.08 ^a	2.46±0.07 ^a	2.67±0.21 ^a
Calorie intake (kcal/day)	15.12±0.38 ^b	12.36±0.37 ^a	11.20±0.32 ^a	12.59±0.98 ^a

LF: low fat, HF: high fat, HFDJ: HF containing *Doenjang*, HFSS: HF containing steamed soybean.

Results are given as the mean ± SEM (n = 11–12). Means in the same row that do not share the same superscript are significantly different (one-way ANOVA followed by Duncan multiple range test, $P < 0.05$).

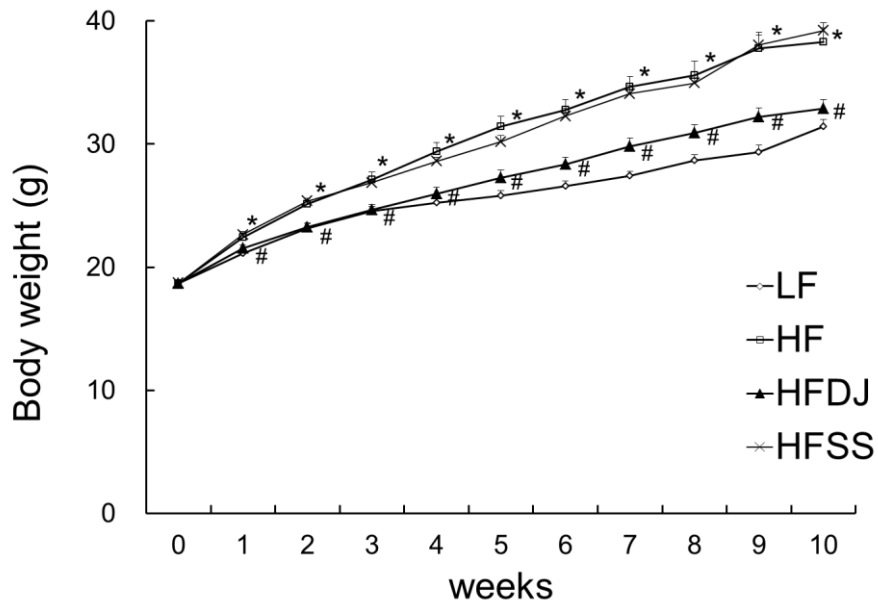


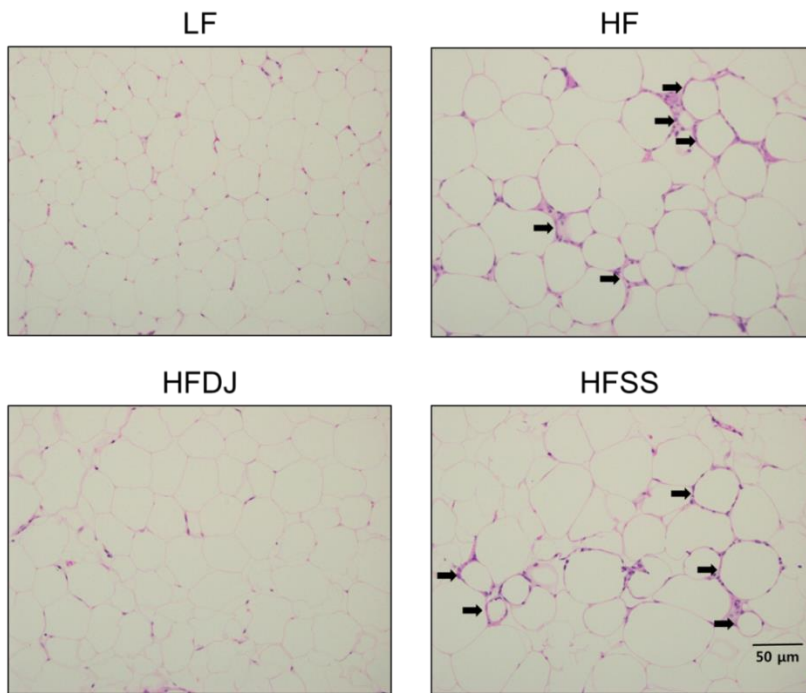
Figure 2. Body weight change of mice fed a high fat diet (HF) and HF containing *Doenjang* (HFDJ) or steamed soybean (HFSS).

Data are presented as means \pm SEM (n = 10–11). * HF group vs. LF group ($P < 0.05$). # HFDJ group vs. HF group ($P < 0.05$). Data were analyzed by one-way ANOVA followed by Duncan multiple range test.

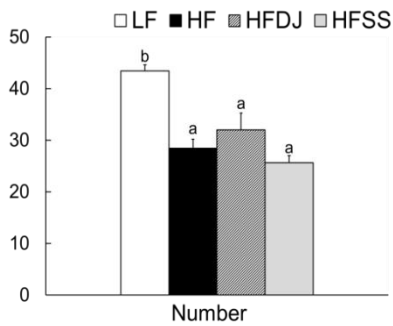
Effects of Doenjang on adipose tissue morphology of mice fed a high fat diet

The microscopic examination of H&E staining of adipose tissue exhibited adipocyte hypertrophy and inflammation in mice fed an HF diet (**Fig. 3A**). We observed significantly increased size and decreased number of adipocytes in mice fed an HF diet compared to those in mice fed an LF diet, but there were no significant differences in size and number of adipocytes among the HF diet-fed groups (**Fig. 3B&C**). Thus, consumption of *Doenjang* and steamed soybean had no effect on the adipocyte hypertrophy. CLS, mainly existed in inflammatory adipose tissue, were less observed in the adipose tissue of the HFDJ and the LF groups compared to the HF group and the HFSS groups (**Fig. 3A**).

(A)



(B)



(C)

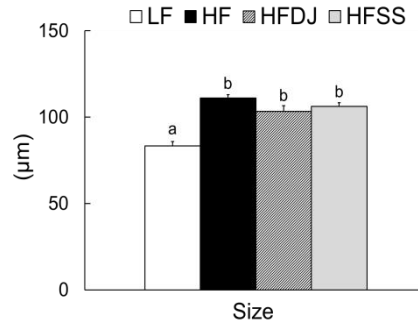


Figure 3. Adipocyte morphology of mice fed a high fat diet (HF) and HF containing *Doenjang* (HFDJ) or steamed soybean (HFSS).

(A) Representative H&E staining of adipose tissue sections (magnification 200X). Scale bar represents 50 μm. Crown-like structures are indicated with the black arrow. (B) Number of adipocytes per field of observation. (C) Size of adipocytes. Each bar represents the means \pm SEM ($n = 5$) and bars that do not share the same superscript are significantly different (one-way ANOVA followed by Duncan multiple range test, $P < 0.05$).

Effects of Doenjang on oxidative stress in adipose tissue of mice fed a high fat diet

Doenjang has been shown to have anti-oxidant activity *via* regulation of enzyme levels involved in cellular oxidative stress. ROS can be generated by various intracellular enzymes. We observed that mRNA levels of p40^{phox}, a subunit of NOX which is involved in superoxide generation (14), were significantly increased in mice fed an HF diet. Consumption of *Doenjang*, but not steamed soybean, significantly reduced mRNA levels of p40^{phox} (**Fig. 4A**). To investigate anti-oxidants levels which defense against ROS, we measured catalase, an anti-oxidative stress enzyme, and HO-1, a stress-inducible protein. The protein levels of catalase were significantly lower in the HFDJ group compared to those of the HF and the HFSS groups (**Fig. 4B**). The mRNA level of HO-1 was significantly induced in mice fed an HF diet, while it was reduced by consumption of *Doenjang* (**Fig. 4C**). Although we observed the lower HO-1 protein levels in the HFDJ group than in the HF group, the difference was not significant. To sum up, we anticipated that consumption of *Doenjang* reduces ROS level *via* reducing ROS activity, which resulted in the decrease in expressions of catalase and HO-1 which defense against ROS.

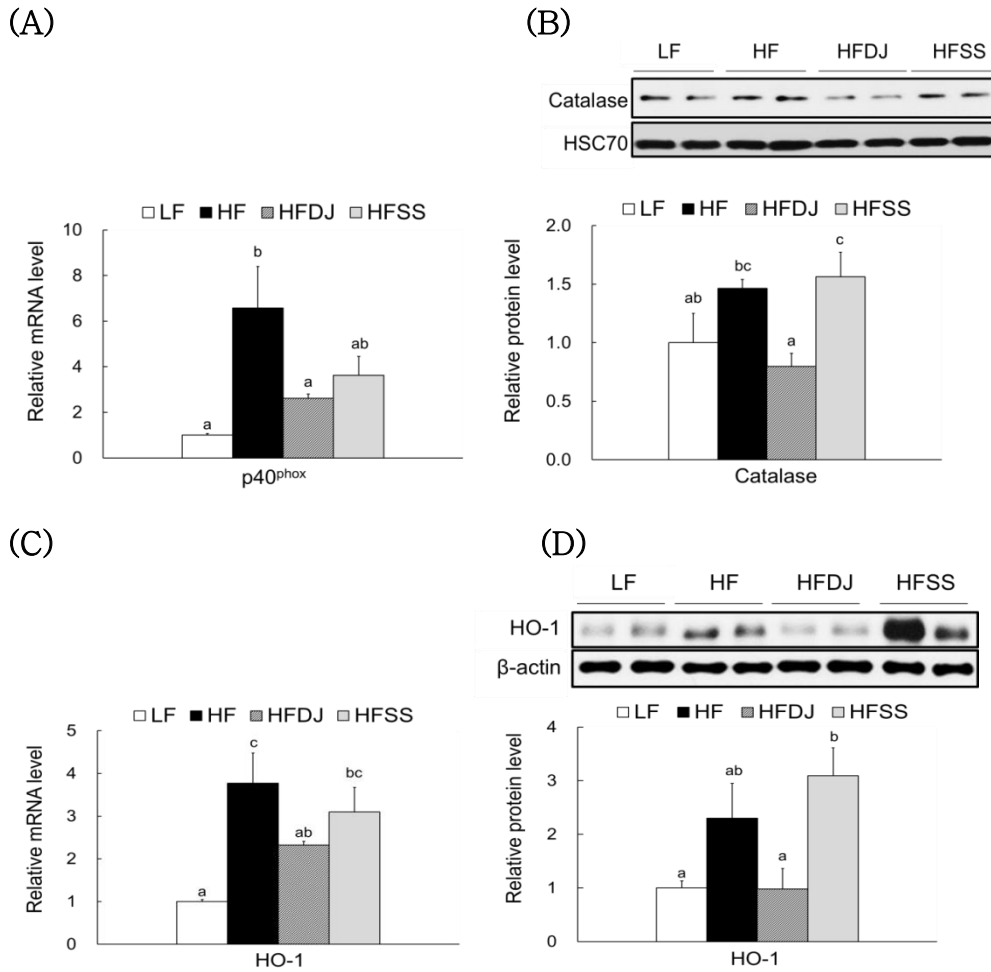


Figure 4. Relative level of oxidative stress markers in adipose tissue of mice fed a high fat diet (HF) and HF containing *Doenjang* (HFDJ) or steamed soybean (HFSS).

(A) Relative mRNA level of p40^{phox}/RPL19 was determined by qPCR (n = 3-4). (B) Relative protein level of catalase/HSC70 was determined by immunoblotting (n = 4). (C) Relative mRNA level of HO-1/RPL19 was determined by qPCR (n = 3-4). (D) Relative protein level of HO-1/β-actin was determined by immunoblotting (n = 4). Each bar represents the means ± SEM and bars that do not share the same superscript are significantly different (one-way ANOVA followed by Duncan multiple range test, $P < 0.05$).

Effects of Doenjang on adipose tissue inflammation of mice fed a high fat diet

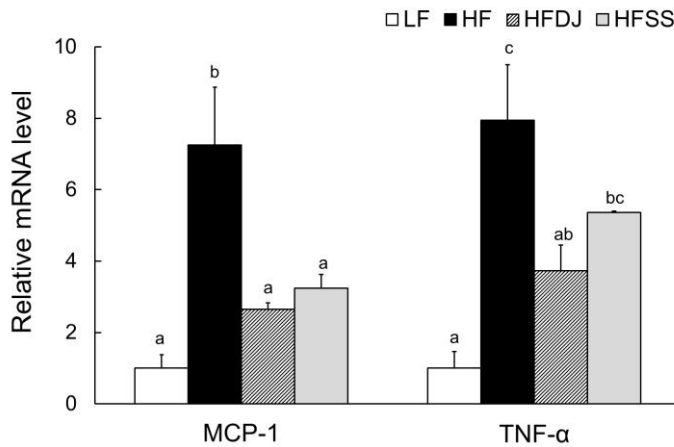
Because we observed that consumption of *Doenjang* reduced CLS formation in adipose tissue of HF diet-fed mice, we assessed expression of pro-inflammatory and anti-inflammatory genes in adipose tissue. Hypertrophic adipocytes stimulate the release of pro-inflammatory adipokines and macrophage infiltration. The mRNA levels of pro-inflammatory genes were significantly increased in the HF group compared to the LF group (**Fig. 5A**). *Doenjang* consumption significantly reduced the mRNA levels of TNF- α , MCP-1, while steamed soybean consumption significantly reduced the mRNA levels of MCP-1 but not TNF- α . The gene expression of adiponectin, an anti-inflammatory adipokines, was significantly decreased in the HF group, while it was significantly increased in both the HFDJ and the HFSS groups (**Fig. 5B**). Furthermore, there was a strong negative correlation between TNF- α and adiponectin mRNA levels (**Fig. 5C**).

Consistent with CLS incidence, which formed by infiltrated macrophages, the mRNA levels of CD68, a total macrophage marker (71) and CD11c, a surface marker of M1 type (72) were significantly increased in the HF group compared to the LF group (**Fig 6A**). Consumption of *Doenjang* significantly reduced mRNA expressions of these genes involved in infiltration and phenotype of macrophage, but in the HFSS groups CD11c only significantly reduced. Especially, MCP-1 is one of the chemokine, which regulates infiltration of

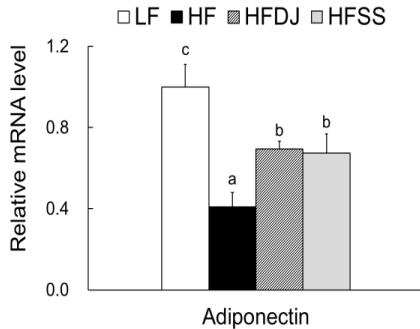
macrophages (73). In this study, there was a strong positive correlation between MCP-1 and CD68 mRNA levels (**Fig. 6B**).

Because TNF- α induces pro-inflammatory adipokine secretion *via* activation of JNK signaling (74), we investigated whether consumption of *Doenjang* modulates JNK activation in adipose tissue of mice fed an HF diet. Phosphorylated JNK-1 level was significantly increased in the HF group compared to the LF group (**Fig. 7**). Consumption of *Doenjang* and steamed soybean tended to down-regulate p-JNK1 level in adipose tissue of mice fed an HF diet.

(A)



(B)



(C)

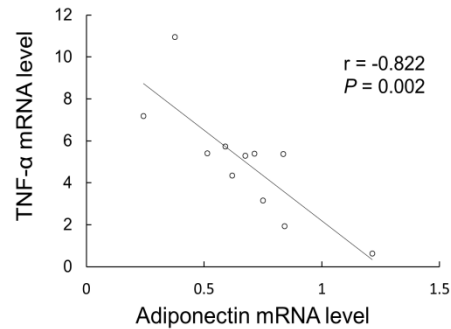
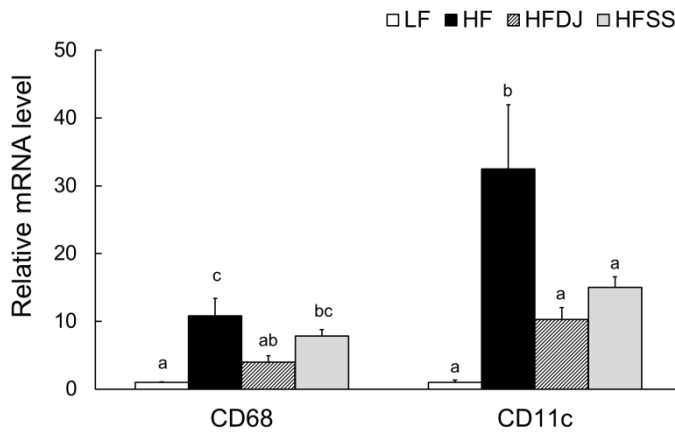


Figure 5. Relative expression of inflammation markers in adipose tissue of mice fed a high fat (HF) and HF containing *Doenjang* (HFDJ) or steamed soybean (HFSS).

Relative mRNA expression of genes involved in (A) pro-inflammatory and (B) anti-inflammatory adipokine was determined by qPCR and was normalized to RPL19. Each bar represents the means \pm SEM ($n = 3-4$) and bars that do not share the same superscript are significantly different (one-way ANOVA followed by Duncan multiple range test, $P < 0.05$). (C) Pearson's correlation between relative adiponectin mRNA expression and relative TNF- α mRNA expression.

(A)



(B)

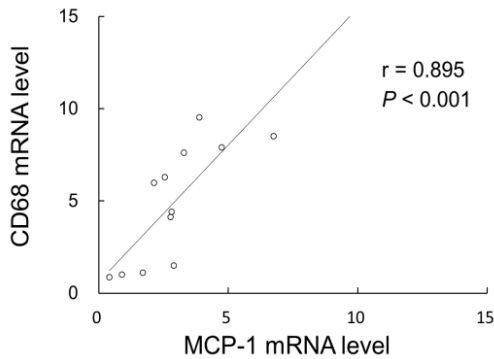


Figure 6. Relative expression of macrophage markers in adipose tissue of mice fed a high fat (HF) and HF containing *Doenjang* (HFDJ) or steamed soybean (HFSS).

Relative mRNA expression of genes involved in (A) macrophage markers was determined by qPCR and was normalized to RPL19. Each bar represents the means \pm SEM ($n = 3-4$) and bars that do not share the same superscript are significantly different (one-way ANOVA followed by Duncan multiple range test, $P < 0.05$). (B) Pearson's correlation between relative MCP-1 mRNA expression and relative CD68 mRNA expression.

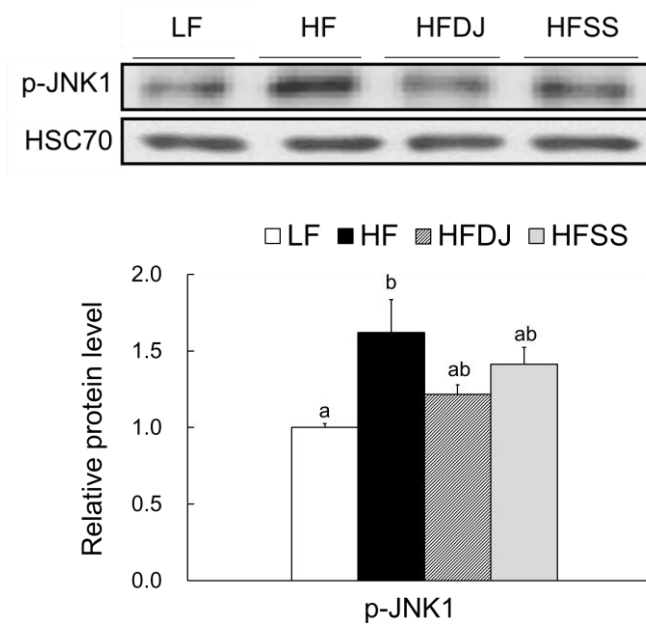


Figure 7. Activation of inflammatory signaling in adipose tissue of mice fed a high fat (HF) and HF containing *Doenjang* (HFDJ) or steamed soybean (HFSS).

Relative protein level of p-JNK1/HSC70 was determined by immunoblotting. Each bar represents the means \pm SEM ($n = 4$) and bars that do not share the same superscript are significantly different (one-way ANOVA followed by Duncan multiple range test, $P < 0.05$).

Effects of Doenjang on adipose tissue fibrosis of mice fed a high fat diet

High fat feeding significantly induced mRNA levels of TGF- β 1 and COL1A1, genes involved in fibrosis development (**Fig. 7**). Consumption of *Doenjang* significantly reduced mRNA levels of TGF- β 1, but not COL1A1. Although the consumption of *Doenjang* was not shown to be significant difference, tends to down-regulate mRNA levels of COLA1, compared to the HF group. No inhibitory effects were observed in mice fed an HFSS diet.

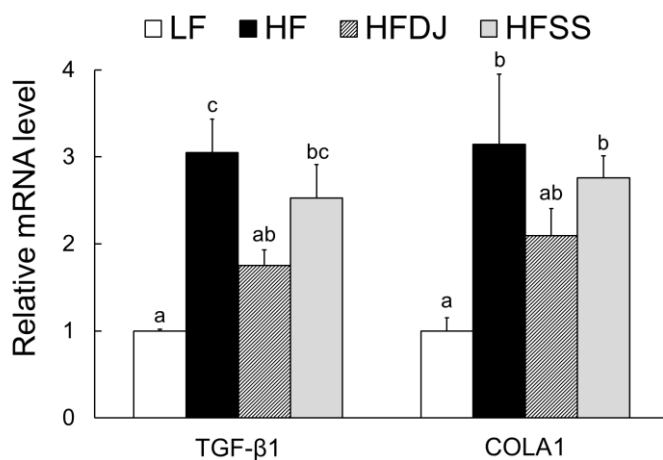


Figure 8. Relative expression of fibrosis markers in adipose tissue of mice fed a high fat (HF) and HF containing *Doenjang* (HFDJ) or steamed soybean (HFSS).

Relative mRNA expression of genes involved in fibrosis was determined by qPCR and was normalized to RPL19 (n = 3-4). Each bar represents the means \pm SEM and bars with different superscripts are significantly different (one-way ANOVA followed by Duncan multiple range test, $P < 0.05$).

Discussion

It has been suggested that numerous chemokines and adipokines secreted from adipose tissue are the main contributors of systemic inflammation in obesity. Under conditions of obesity, different adipose tissue cells, including adipocytes, macrophages and stromal fraction vascular cells lead to the production and secretion of adipokines (3). Although anti-obesity effects of *Doenjang* have been reported by previous studies (37), (75), this study particularly evaluated the protective effects of *Doenjang* on oxidative stress, inflammation and fibrosis in adipose tissue using high fat diet-induced obese mice model.

In the present study, *Doenjang* protects against oxidative stress *via* inhibiting ROS production and enhancing ROS degradation, which is confirmed by lower mRNA levels of HO-1, a microsomal enzyme induced in response to variety of stimuli such as heavy metals, oxidative stress and cytokines (76), and mRNA levels of p40^{phox}, a subunit of NOX. Elevated oxidative stress has been regarded as a potential contributing factor in the accumulation of immune cells in adipose tissue. Therefore, reducing oxidative stress and inflammation in adipose tissue will positively influence the risk of metabolic syndrome (13). In this regard, genistein suppressed the expressions of the p22^{phox}, a NOX subunit in a concentration- and

time-dependent manners in aortic endothelial cells from stroke-prone spontaneously hypertensive rats (77).

It is of note that TNF- α mRNA levels were significantly reduced in the HFDJ group compared to the HF group. TNF- α is mainly overproduced by macrophages and contributes to dysregulated production or secretion of adipokines in adipose tissue (3). Furthermore, the mRNA expressions of MCP-1, CD68 and CD11c were reduced in the HFDJ group compared to the HF group. It means that *Doenjang* suppresses secreting pro-inflammatory adipokines and inflammatory signaling from M1 macrophage through preventing macrophage infiltration in adipose tissue during obesity. On the other hand, adiponectin, an anti-inflammatory adipokine, shows protective actions on the development of various obesity-linked diseases and it is negatively regulated by TNF- α in adipose tissue (78). As expected, we observed that the mRNA expression of adiponectin was significantly increased in the HFDJ group compared to the HF group. These results suggest that *Doenjang* may alleviate obesity-linked metabolic disorders by regulation of altered adipokine production. According to a previous study, incubation with genistein reduced IL-6 and MCP-1 levels in human intestinal epithelium cell treated with a cocktail of pro-inflammatory stimuli, composed of IL-1 β , TNF- α , IFN- γ and LPS (79).

Inflammation are tightly associated with an excess synthesis of fibrillar collagens and a failure of degradation of these proteins in adipose tissue (7). Abnormal collagen deposition can exhibit

considerable phenotype modulation with deleterious effects on tissue homeostasis and function (24). TGF- β 1, induced by macrophage, is an important regulator of fibrosis by enhancement of alpha smooth muscle actin and COL1A1 (80). We observed that consumption of *Doenjang* reduced mRNA levels of fibrosis markers, suggesting that the inhibitory effect of *Doenjang* on adipose tissue fibrosis in obesity. Similarly, genistein, a major isoflavone in *Doenjang*, has been shown to exert anti-fibrotic effects by suppressing hepatic stellate cell proliferation and alpha smooth muscle expression in response to platelet-derived growth factor (62).

In the present study, we observed the resolution of inflammation and fibrosis without changes in number and size of adipocyte by consumption of *Doenjang*. Similarly, a previous study reported that dipeptidyl peptidase-4, which degrades numerous peptides and chemokines, reduced expression of inflammation markers without affecting number and size of adipocyte (81). It has been shown that increased ECM components may interfere with adipose mass expansion in metabolically dysfunctional adipose tissue (1). Therefore, at least in the present study, *Doenjang* may exert its anti-obesity effects by inhibiting further crosstalk between adipocytes and macrophages, which comes after adipocyte hypertrophy. These qualitative changes in enlarged adipocytes by *Doenjang* may contribute to the transition of adipocytes from a metabolically dysfunctional phenotype to a metabolically functional phenotype.

We observed the stronger anti-obesity effect of *Doenjang*

compared to steamed soybean in diet-induced obese model. Furthermore, this is the first study to report that *Doenjang* exhibited anti-oxidative and anti-inflammatory effects in the adipose tissue of obese mice. Consistent with previous study, we observed that isoflavone and phenolic compounds content of *Doenjang* is higher than soybean. Total content of isoflavone glucoside forms in an HFDJ diet and an HFSS diet was 5.76 and 336.96 mg/kg diet, respectively. In contrast, total content of isoflavone aglycone form in an HFDJ diet and an HFSS diet was 152.64 and 15.21 mg/kg diet, respectively. Especially, the content of genistein in an HFDJ diet (73.44 mg/kg diet) was about 10-times higher than that in an HFSS diet (7.02 mg/kg diet). Total content of polyphenols in an HFDJ diet and an HFSS diet was 2244.96 and 359.19 mg TAE/kg diet (based on **Table 1** and **Table 2**). Therefore, the increase in isoflavone aglycone and total polyphenols content during fermentation is believed to be responsible for the high pharmacological activity of *Doenjang*.

In this study, we only measured content of bioactive compounds in *Doenjang* and steamed soybean. Thus, additional research on activity of bioactive compounds of *Doenjang* and steamed soybean is needed.

In conclusion, dietary consumption of *Doenjang* ameliorated adipose tissue inflammation, oxidative stress and fibrosis in high fat-induced obesity model (**Fig. 9**). On the contrary, steamed soybean did not exert these beneficial effects in adipose tissue of obese mice. Considering the potential to restore altered inflammatory mechanism, oxidative stress and fibrosis in adipose tissue, *Doenjang* may lead to

the reduced risk of metabolic syndrome development in obese subjects.

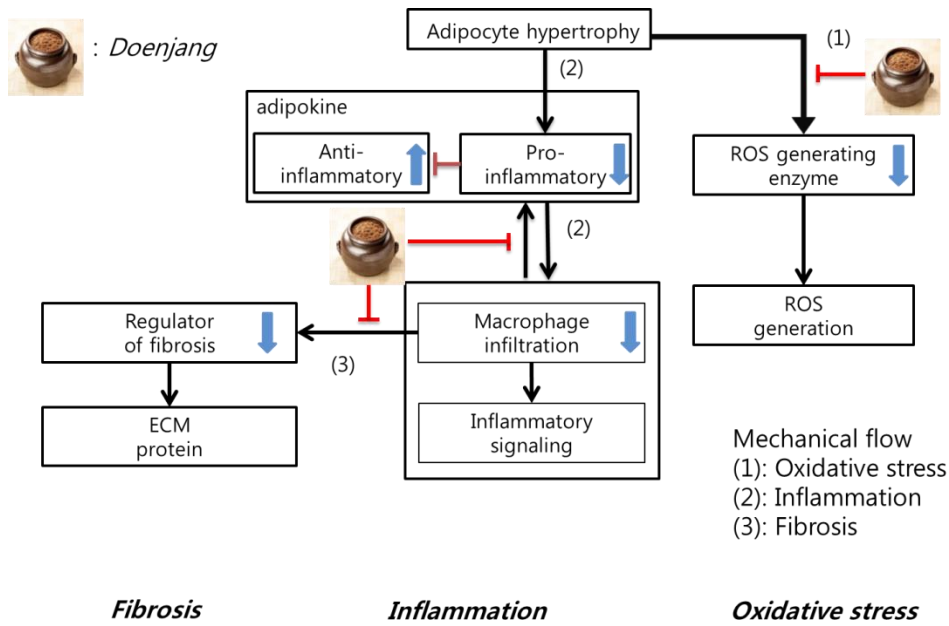


Figure 9. Potential mechanism of anti-inflammatory, anti-fibrotic and anti-oxidative stress action of *Doenjang*.

During development of obesity, adipocyte size increase (hypertrophy). Hypertrophic adipocytes cause inflammation and oxidative stress. *Doenjang* alleviates oxidative stress *via* down-regulating NOX activity. In addition, *Doenjang* suppresses the release of pro-inflammatory adipokines such as TNF- α and MCP-1. Furthermore, due to the decreased CD68 and CD11c levels in the adipose tissue of *Doenjang* consumption group, we could reveal that *Doenjang* has shown to be effective in preventing macrophage infiltration. Besides, *Doenjang* attenuates fibrosis, induced by infiltrated macrophages, *via* suppressing TGF- β 1 level and accumulation of ECM proteins.

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국문 초록

고지방식이를 섭취한 마우스 지방조직에서 된장이 산화스트레스 및 염증에 미치는 영향

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남예림

최근 낮은 수준의 만성 염증상태로 간주되고 있는 비만은 대사성 증후군과 상관관계가 있다. 특히, 지방조직은 인슐린 저항성을 악화시키는 염증성 사이토카인의 분비를 통해 비만의 전신성 염증상태를 초래하는 주요한 기관으로 여겨지고 있다. 된장은 한국 전통 발효 장류 음식으로서 혈액과 간에서 항비만, 항염증 효과가 보고되고 있다. 그러나 지방조직이 비만 관련 염증 반응에 기여하는 중요한 기관임에도 불구하고 지방조직에서의 된장의 항비만, 항염증 효과를 연구한 선행연구가 없었다. 그러므로 본 연구는 비만유도모델의 지방조직에서의 된장의 항염증 및 항산화 효과를 보고자 한다. 6주령의 수컷 C57BL/6J 마우스를 각각 저지방식이 (LF), 고지방식이 (HF: 45% 지질 and 1% 콜레스테롤), 고지방식이에 된장 함유한 식이 (HFDJ: 14.4% 동결 건조한 된장이 포함된 식이) 및 고지방식이에 찐대두 함유한 식이(HFSS: 11.7% 동결 건조한 찐대두가 포함된 식이)를 11주간 공급하였다. 고지방식이에 된장 함유식이를 섭취한 마우스는 고지방식이그룹에 비해 체중이 16%, 지방조직

무게는 19% 유의적으로 낮았다. 고지방식이를 한 그룹들 간에서는 지방 세포 크기와 수에서 유의적인 차이가 없었으나, 된장의 섭취는 된장의 섭취는 지방 조직 내 왕관모양 구조의 발생 정도를 완화시켰다. 마찬가지로, 저지방식이그룹과 고지방식이에 된장을 함유한 식이 그룹에서 고지방식이 그룹에 비하여 산화스트레스 지표 (heme oxygenase-1, p40^{phox}), 염증성 아디포카인 (tumor necrosis factor alpha, macrophage chemoattractant protein-1), 대식세포 지표 (CD68, CD11c)와 섬유화 증 지표 (transforming growth factor beta 1)의 mRNA 수준이 유의적으로 감소하였다. 항염증성 아디포카인인 아디포넥틴의 유전자 발현은 고지방식이 그룹에 비해 고지방식이에 된장 함유한 식이그룹과 대두 함유한 식이그룹에서 유의적으로 증가하였다. 이러한 결과들은 된장이 지방조직의 염증 신호전달체계를 억제함으로써 비만에서의 전신염증과 산화스트레스를 완화시킬 것으로 사료된다. 된장과 달리 찐대두 식이에서는 지방조직의 염증 및 산화스트레스에 효과적이지 않았다. 이는 발효와 숙성과정에서 발생한 생리활성 물질함량이 된장의 이로온 효과에 기여한 것으로 판단된다.

주요어: 된장, 비만 마우스, 산화스트레스, 염증, 지방조직

학 번: 2013-21501